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A Guide to Computational Tools and Design Strategies for Genome Editing Experiments in Zebrafish Using CRISPR/Cas9

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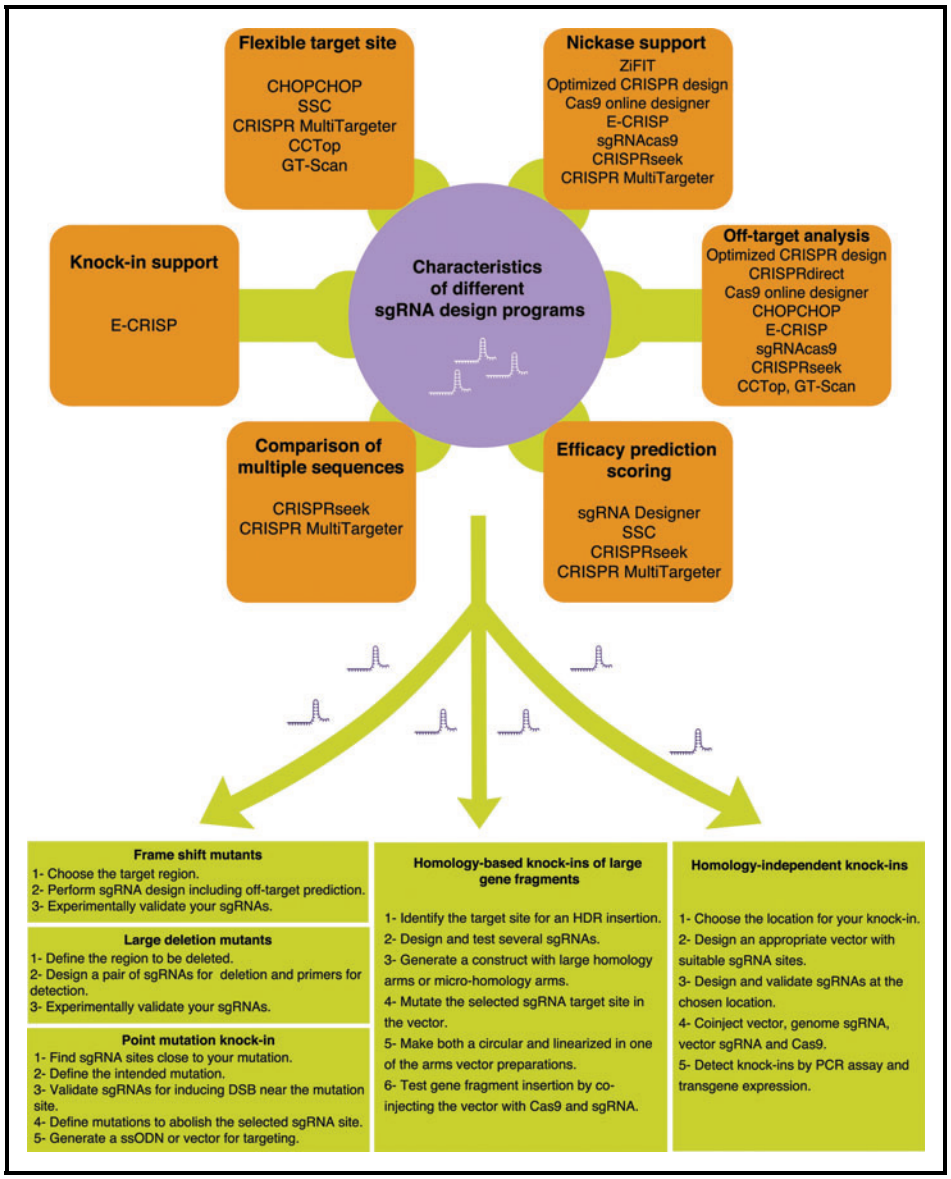

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Abstract

The development of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology for mainstream biotechnological use based on its discovery as an adaptive immune mechanism in bacteria has dramatically improved the ability of molecular biologists to modify genomes of model organisms. The zebrafish is highly amenable to applications of CRISPR/Cas9 for mutation generation and a variety of DNA insertions. Cas9 protein in complex with a guide RNA molecule recognizes where to cut the homologous DNA based on a short stretch of DNA termed the protospacer-adjacent motif (PAM). Rapid and efficient identification of target sites immediately preceding PAM sites, quantification of genomic occurrences of similar (off target) sites and predictions of cutting efficiency are some of the features where computational tools play critical roles in CRISPR/Cas9 applications. Given the rapid advent and development of this technology, it can be a challenge for researchers to remain up to date with all of the important technological developments in this field. We have contributed to the armamentarium of CRISPR/Cas9 bioinformatics tools and trained other researchers in the use of appropriate computational programs to develop suitable experimental strategies. Here we provide an in-depth guide on how to use CRISPR/Cas9 and other relevant computational tools at each step of a host of genome editing experimental strategies. We also provide detailed conceptual outlines of the steps involved in the design and execution of CRISPR/Cas9-based experimental strategies, such as generation of frameshift mutations, larger chromosomal deletions and inversions, homology-independent insertion of gene cassettes and homology-based knock-in of defined point mutations and larger gene constructs.

THE RECENT EXPLOSION in the field of genome editing technologies such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 enables precise and efficient manipulation of genomes in cells and model organisms.[1] In particular, use of CRISPR/Cas9 enables more rapid genome editing reagent development than TALENs, although TALENs maintain some other advantages, such as a lower off-target activity and potentially higher rates of cleavage.[1,2] The type IIB CRISPR/Cas9 system functions in bacteria to protect them against molecular pathogens by incorporating small pieces of pathogen DNA into CRISPR loci and expressing them as RNA, which in complex with Cas9 cuts the homologous DNA.[3] The system has been engineered for experimental use by fusing crRNA and tracrRNA into single-guide RNA (sgRNA) that can target a defined sequence in complex with Cas9, which

FIG. 1. Classification of sgRNA design software tools and conceptual steps for different types of genome editing strategies. In this figure, we classify a number of sgRNA design tools, which can be applied to zebrafish: ZiFiT,[15] Cas9 Design,[16] Optimized CRISPR Design, CRISPRdirect,[17] Cas9 Online Designer, CHOPCHOP,[18] E-CRISP,[10] sgRNAcas9,[19] sgRNA Designer,[7] SSC,[6] CRISPRseek,[8] CRISPR MultiTargeter,[9] CCTop,[20] and GT-Scan.[21] We classify these tools according to the availability of flexible target site definition; support for design of sgRNA pairs for Cas9 nickase-based targeting; off-target analysis availability; calculation of efficacy score based on the sequence; ability to design sgRNAs in the context of multiple sequences; and support for knock-in strategy design. These sgRNAs can then be used for various experimental purposes such as generating frameshift or large deletion mutants, point mutation knock-in, homology-independent knock-ins, and homology-based knock-ins of large gene fragments. Each of the experimental strategies is briefly described by the main steps involved. CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-stranded break; HDR, homology-dependent repair; sgRNA, single-guide RNA; ssODN, single-stranded oligodeoxynucleotide. Color images available online at www.liebertpub.com/zeb

becomes in effect a sequence-specific nuclease.[4] Inside cells, such cleavage results in insertions or deletions (indels) due to activities of DNA repair systems.

The pace of research in this area is really astounding, which makes it difficult for busy investigators to remain current with all the important developments. Similarly, researchers just starting with CRISPR/Cas9 may be overwhelmed by the wide variety of computational design tools and experimental protocols. The good news is that most of these tools work, but model species differ in how they can be manipulated and in availability of specific kinds of modifications. Therefore, there is a need for reviews and how-to explanatory articles, which can quickly direct readers to the most suitable techniques and approaches. Classroom teaching on genome editing is also becoming popular at scientific conferences and workshops. The authors of this article ran a workshop entitled ''Computational tools for genome editing using CRISPR single-guide RNAs'' at the North Atlantic Zebrafish Research Symposium in Halifax, Nova Scotia, in June 2015, which was formatted as a tutorial (available at http://learncrispr.blogspot.com/2015/11/NAZRS-CRISPR-workshop.html).

Optimization of CRISPR/Cas9-based genome editing experiments should focus on careful selection of sgRNA sites. Software tools are helpful in this process, but their diversity can complicate choosing the most suitable software. In this study, we provide a detailed guide to the functions, strengths, and weaknesses of individual sgRNA design software programs and then focus on the procedures for particular types of genome editing experiments in zebrafish. The programs supporting design of sgRNAs for the zebrafish genome are listed in the Figure 1, grouped by their most important properties and further discussed in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/zeb). The first aspect of sgRNA design software is the degree of flexibility in sgRNA target site definition. The Cas9 D10A (nickase) mutant cleaves only one DNA strand and requires a pair of sgRNAs for inducing a double-stranded break.[5] The ability to run paired sgRNA designs for Cas9 nickase (nickase support) is another aspect of target site definition, which may have relevance in zebrafish. Another important aspect of sgRNA design software tools is off-target analysis, which can help choose more specific sgRNAs. Some software tools are designed to predict efficacies of sgRNAs.[6,7] Additional features of sgRNA design programs are the ability to suggest several approaches based on multiple sequences[8,9] and knock-in support.[10]

We discuss design aspects for applications employing the CRISPR/Cas9 system in the Supplementary Data. Generation of frameshift mutants in protein-coding genes requires the user to choose the target region, design sgRNAs, check off-target predictions, and then experimentally validate the resulting sgRNAs. Generation of deletion mutants is a doubling of the strategy for inducing single-site mutations, but they can be detected by single-step PCR.[11] Generation of small defined mutations has been demonstrated in zebrafish, but most instances of mutation knock-ins contain indels at the sgRNA target site.[12] To produce zebrafish with specific mutations, one needs to define the mutation and identify appropriate proximally positioned sgRNA target sites, validate several sgRNAs, and then design a donor template molecule for homology-dependent repair. The current options for homology templates are single-stranded oligodeoxynucleotides[12,13] and plasmids with flanking sites to be cut by Cas9 programmed with the same or an additional sgRNA.[14] We also discuss the design strategies for larger insertions using CRISPR/Cas9 technology and alternative plasmid donors. Overall, our intention is to describe essential steps of CRISPR/Cas9-based genome editing experiments in detail with an emphasis on computational tools and conceptual design.

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Disclosure Statement

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